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Cytolysis of Malignant Glioma Cells by Lymphokine-Activated Killer Cells Combined With Anti-CD3/Antiglioma Bifunctional Antibody and Tumor Necrosis Factor-α

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With the aim of developing an effective immunotherapy for malignant glioma, glioma cells were incubated with tumor necrosis factor-α (TNF-α) to increase their susceptibility to lysis by lymphokine-activated killer (LAK) cells. Treatment with exogenous TNF-α induced the expression of intercellular adhesion molecule-1 (ICAM-1) on the surface of glioma cells. In addition, the cytolytic activity of LAK cells toward exogenous TNF-α-treated glioma cells was significantly greater than LAK cell activity toward untreated glioma cells. This increase in cytolytic activity was blocked by anti-ICAM-1 monoclonal antibodies (MAb). Furthermore, co-treatment with a bifunctional antibody (BFA) composed of anti-CD3 (UCHT1) and antiglioma (G-22) antibodies synergistically increased the cytolytic activity of LAK cells towards TNF-α-treated glioma cells. These results indicate that a combination of exogenous TNF-α and anti-CD3/antiglioma BFA may provide an effective modified adoptive immunotherapy for patients with malignant glioma.

KEY WORDS: glioma, bifunctional antibody, TNF-α, ICAM-1

INTRODUCTION

Lymphokine-activated killer (LAK) cells generated from peripheral blood lymphocytes (PBLs) treated with interleukin-2 (IL-2) are known to kill a wide variety of allogeneic and autologous tumor cells [1–3]. Although adoptive immunotherapy with intratumoral infusion of LAK cells has already been used for patients with malignant glioma [2,4], the clinical results have been disappointing [5,6]. This may be because glioma cells produce immunosuppressive factors such as transforming growth factor-β (TGF-β) [6,7] or because glioma cells do not effectively interact with LAK cells as they lack no major histocompatibility complex (MHC) antigens or adhesion molecules required for LAK cell recognition of target cells. These possibilities have given rise to attempts to ensure that LAK cells reach their target tumor cells in vivo more effectively [8–10].

LAK cells exhibit MHC-unrestricted cytotoxicity. While the mechanism underlying the MHC-unrestricted cytotoxicity of LAK cells remains unclear, it is thought that expression of adhesion molecules allows LAK cells to interact with target cells effectively [11,12] and/or to increase the susceptibility of target cells to LAK cell-mediated cytotoxicity [13]. Nitta et al. [10] and Obukhov et al. [14] have demonstrated that treatment with a bispecific antibody, which we call bifunctional antibody (BFA), composed of an anti-CD3 monoclonal antibody (MAb) chemically conjugated with an antiglioma (MAb, not only increased the lysis of target cells but also induced cross-

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linking of effector to target cells. On the other hand, it has already been reported that tumor necrosis factor-α (TNF-α) possesses antitumor activity against glioma cells [15]. Furthermore, TNF-α has the potential to induce the expression of some adhesion molecules on the surface of glioma cells. In this study, we modified adoptive LAK cell immunotherapy by combining it with exogenous TNF-α and BFA.

MATERIALS AND METHODS

Glioma Cell Lines

U251-MG, a human glioma cell line resistant to natural killer (NK) cell-mediated cytolysis [14], and KMS-II, a human ependymoma cell line, were used. Both cell lines were cultured in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS), 0.1 mM nonessential amino acids, 5 mM L-glutamine, and antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml).

Cytokines

Recombinant human TNF-α (specific activity 2.2×10^6 U/mg protein) was obtained from the Asahi Chemical Industry Co., Ltd., (Tokyo, Japan). Recombinant human IL-2 was obtained from the Takeda Pharmaceutical Co., Ltd. (Osaka, Japan).

Monoclonal Antibodies

UCHT1 is a murine MAb against human CD3-antigen expressed on the surface of T lymphocytes (Imperial Cancer Research Technology Ltd., London, UK). G-22 MAb was prepared in our laboratory and specifically reacts with a surface antigen (G-22) of human glioma cells. The specificity of this MAb and biochemical characterization of the antigen it recognizes have previously been described [16]. Anti-ICAM-1 MAb was purchased from British Biotechnology Products Ltd. (Oxford, UK), and FITC-conjugated goat antimouse IgG MAb was purchased from the Medical and Biological Laboratories Co., Ltd. (Nagoya, Japan).

Generation of LAK Cells

Heparinized blood was obtained from healthy allogeneic donors. Mononuclear cells were separated by standard Ficoll-Hypaque gradient centrifugation. Cells collected at the gradient interface were washed twice with phosphate-buffered saline (PBS) to obtain PBLs. We resuspended the PBLs in RPMI 1640 medium containing 10% FCS, 2 mM L-glutamine, antibiotics (streptomycin, 100 μg/ml; penicillin, 100 U/ml), and 10 U/ml of recombinant human IL-2. For this study, we used LAK cells after 5 days in culture. These LAK cells are composed mainly of $CD16^+$ NK cells (data not shown).

Preparation of BFA

G-22-F $(ab')_2$ fragments (5 mg/ml) were reduced with 1.0 mM dithiothreitol (DTT) at room temperature for 30 min to prepare Fab' fragments. Fab' fragments were incubated with an equal volume of 10 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for 30 min to react with an −SH residue, resulting in the generation of Fab'-S-nitrobenzoate. The nitrobenzoic acid derivatives of the G-22-Fab' fragments were separated from excess reagents and other reaction products on a Sephadex G-25 column. UCHT1-F$(ab')_2$ fragments were reduced with 0.5 mM DTT for 30 min and immediately separated from excess reagents on a Sephadex G-25 column. UCHT1-Fab' fragments were then mixed with G-22-Fab'-S-nitrobenzoate at a ratio of 1:1 to prepare the BFA UCHT1-Fab'/G-22-Fab'. The mixture was incubated overnight at room temperature and stored at 4°C for 7 days [17]. Generation of BFA was confirmed by SDS-PAGE under nonreducing conditions (data not shown).

Detection of ICAM-1 on the Surface of Glioma Cells Treated With Exogenous TNF-α by Flow Cytometry

Cells were harvested with 0.05% EDTA and aliquoted at a concentration of 1×10^6 cells/tube. The cells were washed and resuspended in 100 μl of anti-ICAM-1 MAb (1:100 dilution), and then incubated for 60 min at 4°C. Treated cells were washed again and resuspended in 20 μl of FITC-conjugated goat antimouse IgG MAb (1:32 dilution), and were incubated for 60 min at 4°C. After two washes, the cells were resuspended in 1 ml of PBS and analyzed immediately on a flow cytometer.

Cytolytic Activity of LAK Cells Toward Glioma Cells

The cytolytic activity of LAK cells was evaluated using a ^{51}Cr release assay. Ten ml of glioma cells suspended in culture medium (5×10^5 cells/ml) was placed in a Falcon plate (#3042) and incubated at 37°C for 24 hr in a humidified atmosphere of 5% CO_2 and 95% air. The glioma cells were incubated with 20 U/ml of exogenous TNF-α added to the culture medium. After 5 days of incubation, cells were harvested with 0.05% EDTA, washed, resuspended in 500 μl of fresh medium, and incubated with 37 MBq of $Na_2{}^{51}CrO_4$ for 45 min at 37°C. The cells were then washed three times with fresh medium and 5×10^4 cells in 100 μl were distributed in each well of a U-bottomed 96-well plate (Corning, #25850). LAK cells, used effector cells, were added to each well at an effector-to-target cell ratio of 5:1, and the plates were incubated for 4 hr at 37°C. After incubation, supernatants were collected from each well, and released ^{51}Cr was measured in a gamma counter. Specific lysis was calculated according to the following formula:

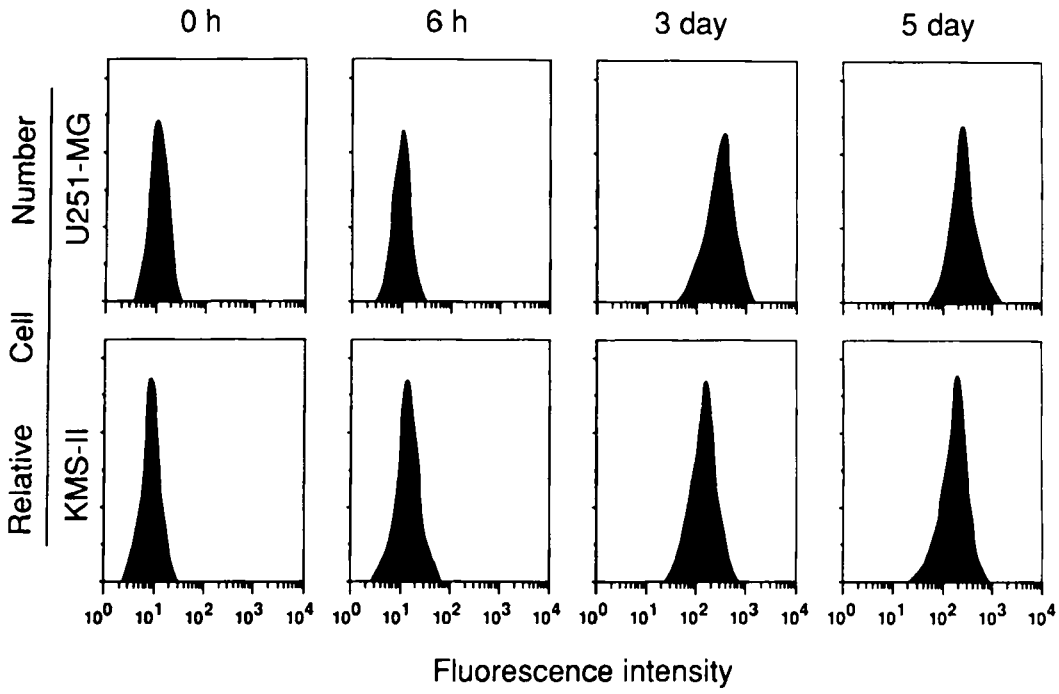

Fig. 1. Expression of ICAM-1 in U251-MG and KMS-II cells treated with exogenous TNF-α. The number of positive cells and fluorescence intensity was measured by flow cytometry.

% Cytolysis = [(experimental cpm-spontaneous cpm)/(total cpm-spontaneous cpm)] × 100.

Combined Effect of Exogenous TNF-α and BFA on Cytolytic Activity of LAK Cells

To investigate the combined effect of LAK cells and BFA on glioma cells pretreated with exogenous TNF-α, BFA (5 μg/ml) was added to culture medium and incubated with LAK cells for 30 min at room temperature before addition to target glioma cells. Cytolytic activity was evaluated by the same procedure described above.

RESULTS

Induction of ICAM-1 on Glioma Cells

Untreated U251-MG and KMS-II cells did not express ICAM-1 on the cell surface. However, treatment with exogenous TNF-α (20 U/ml) induced ICAM-1 on the surface of glioma cells (Fig. 1). The expression of ICAM-1 was not detected until 6 hr after TNF-α treatment and reached a maximum at 72 hr.

LAK Cell-Mediated Cytolysis of Glioma Cells Treated With Exogenous TNF-α

When glioma cells were treated with exogenous TNF-α, ICAM-1 was induced on the cell surface. The cytolytic activity of LAK cells toward treated glioma cells was slightly but significantly elevated compared with the activity of LAK cells towards untreated glioma cells (Table I). We investigated the relationship between ICAM-1 expression on the target cell and increased cytolytic activity of LAK cells using an anti-ICAM-1 mAb. When anti-ICAM-1 MAb (10 μg/ml) was added to the medium, the increased cytolytic activity of LAK cells was significantly reduced (Table I). There was no significant difference between the susceptibility of U251-MG and KMS-II cells to LAK cell-mediated cytolysis, with or without exogenous TNF-α treatment.

TABLE I. Effect of Anti-ICAM-1 Monoclonal Antibody†

	Cytolytic activity (%)	
Target cells	Anti-ICAM-1 (−)	Anti-ICAM-1 (+)
U251-MG		
Not treated	17.1 ± 1.9*	16.8 ± 1.4 (NS)
exogenous TNF-α	23.8 ± 2.0*	18.2 ± 1.6 (NS)
KMS-II		
Not treated	18.6 ± 1.0*	18.0 ± 1.6 (NS)
exogenous TNF-α	24.6 ± 2.7*	18.9 ± 1.8 (NS)

† Exogenous TNF-α (20 U/ml) was added to glioma cells, and the cells were incubated for 5 days. After 5 days incubation, LAK cells were added to target glioma cells (E/T = 5), and cytolytic activity was evaluated by a ^{51}Cr release assay. In addition, the correlation between ICAM-1 expression and cytolytic activity was determined using an anti-ICAM-1 monoclonal antibody. Values indicate mean ±SD of four wells. NS, not significant.
* $P < 0.01$.

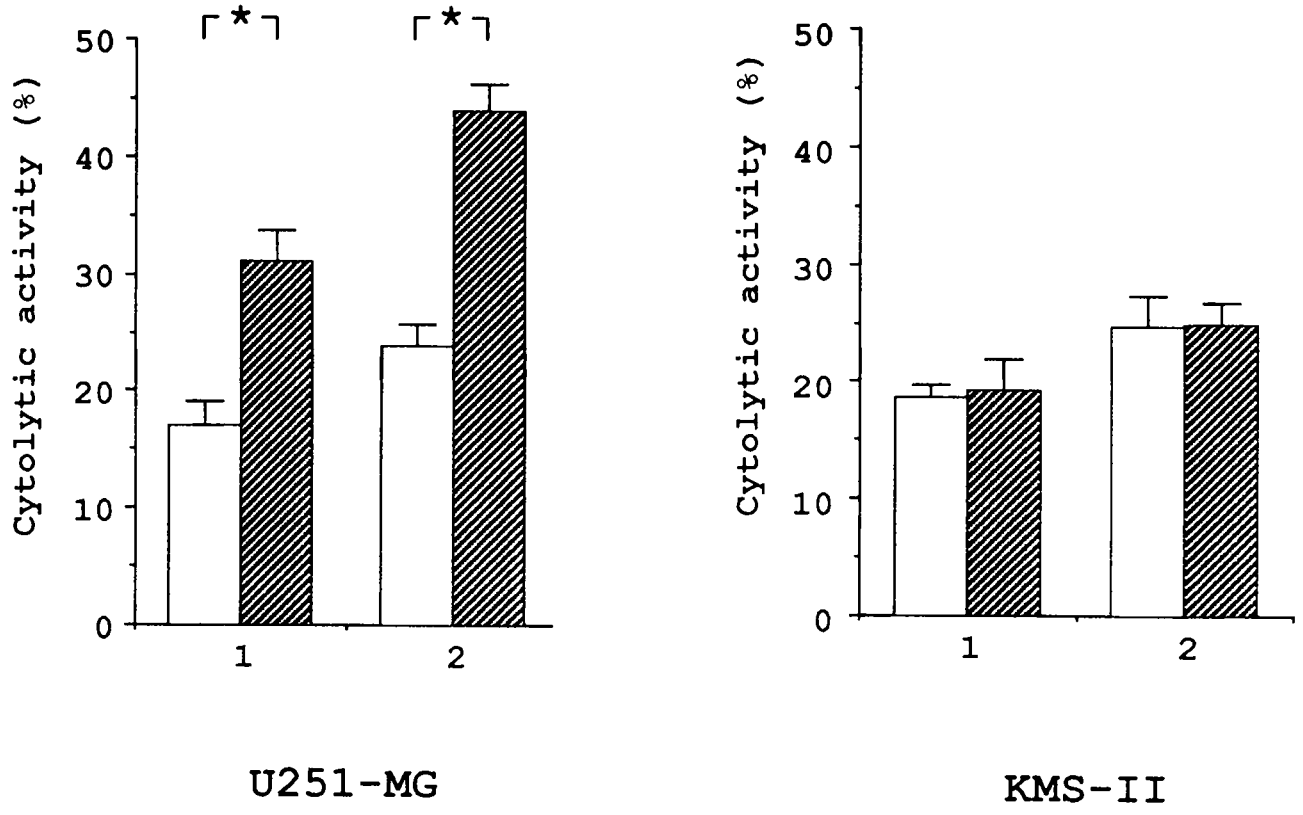

Fig. 2. Combined effect of LAK cells and BFA on glioma cells treated with exogenous TNF-α. BFA was incubated with LAK cells for 30 min. Thereafter, BFA-treated LAK cells were incubated with glioma cells that had been treated with exogenous TNF-α for 5 days. Cytolytic activity was evaluated by a ^{51}Cr release assay 4 hr after adding LAK cells to glioma cells. Open columns, no addition of BFA; shaded columns, addition of BFA (5 μg/ml). Column 1, untreated glioma cells; column 2, glioma cells treated with exogenous TNF-α (20 U/ml). *P <0.01.

Combined Effect of LAK Cells and BFA on Glioma Cells Treated With Exogenous TNF-α

We examined the effectiveness of a combination of LAK cells and BFA on glioma cells treated with exogenous TNF-α. There was no increase in specific lysis when KMS-II cells, which do not possess an antigen recognized by the G-22 MAb, were incubated with LAK cells in the presence of BFA (UCHT1-Fab'/G-22-Fab') (Fig. 2). On the other hand, specific lysis on U251-MG cells, which do possess an antigen recognized by G-22 MAb by LAK cells, was significantly increased in the presence of BFA. This increased lysis was not observed when LAK cells were added to target glioma cells with a mixture of divalent $F(ab')_2$ fragments of UCHT1 and G-22 (data not shown).

DISCUSSION

To be cytolytic, effector LAK cells must bind to target tumor cells. Although the mechanism for killing tumor cells after binding is not well understood, Hook et al. [18] have investigated the cytotoxic effect of LAK cells morphologically and have reported that it is based on local exocytosis of vesicles by the effector cell into small extracellular spaces between the effector and target cells. Recent studies have shown that granules found in LAK cells contain a pore-forming protein (perforin or cytolysin), serine esterase, and tumor necrosis factor (TNF) [19,20]. Perforin produces holes in the target cell plasma membrane, through which as yet unidentified lethal substances are thought to enter target cells where they induce necrosis or apoptosis [21]. Granzyme A or B of serine esterase and TNF-α have also been reported to induce apoptosis in target cells [22]. Our previous studies have demonstrated that the growth of human glioma cells was inhibited by either high doses of TNF-α or by transfection with TNF-α cDNA [23], both of which induced apoptosis. Some investigators have reported that this type of growth inhibition might be related to apoptotic processes induced by the up-regulation of the Fas/APO-1 antigen [19,24]. However, because Fas/APO-1 ligand was not detected in the LAK cells used in this experiment (data not shown), the observed cytolysis could not be related to the Fas/APO-1 ligand.

On the other hand, the susceptibility of glioma cells

to LAK cell-mediated cytolysis increased after treating the cells with exogenous TNF-α. This increased susceptibility was ascribed to the induced expression of ICAM-1 on the surface of target cells, a ligand for the lymphocyte function-associated antigen-1 (LFA-1) expressed on effector LAK cells [25,26]. ICAM-1 expression can be induced by many inflammatory cytokines, such as interferon-γ (IFN-γ), interleukin-1 (IL-1), and TNF-α. In particular, IFN-γ and TNF-α have direct cytotoxic effects on glioma cells. However, poor responses have been reported in a clinical study in which IFN-γ was administered systemically to patients with malignant tumors [27]. By contrast, we have had good results in a clinical study of TNF-α administration to patients with malignant glioma [15]. Therefore, we predict that TNF-α is more effective than IFN-γ, at least, in the treatment of glioma.

In addition, we prepared a bifunctional antibody (BFA) by conjugating anti-CD3 antibody (UCHT1) to antiglioma antibody (G-22) to further increase the growth-inhibitory effect of LAK cells on glioma cells. Similar experiments have been reported by other investigators [10,14,28]. The G-22 MAb we used in this experiment reacts selectively with human glioma tissue, and we have previously used this antibody for clinical radioimaging of gliomas [29]. On the other hand, the anti-CD3 antibody (UCHT1) reacts with cytotoxic T cells (CTLs) and LAK cells. The effect of UCHT1 on the stimulation and modulation of the CD3 complex has already been investigated [30,31]. In our experiments, the presence of BFA greatly increased the cytolysis of glioma cells by LAK cells as expected. Increased cytolysis is speculated to result from the direct linkage of LAK cells to glioma cells through BFA and from CD3 triggering. However, the latter effect appeared to be rather weak, since little T-cell activation was observed even when $F(ab')_2$ fragments of UCHT1 were added to LAK cells.

For clinical trials involving this therapy, we plan to administer LAK cells through an Ommaya reservoir. Our previous experience of adoptive immunotherapy in humans has indicated that severe cerebral edema occurred with IL-2 administration, but not with LAK cell administration. Since the brain is an enclosed cavity separated from the general circulation by the blood–brain barrier, we speculate that local administration of LAK cells and BFA into the tumor will be less toxic than systemic administration. Furthermore, the results of this study indicate that the interaction between LFA-1 and its natural ligand, ICAM-1, play an important role in the adhesion between LAK cells and glioma cells. Also, even NK cell-dominant LAK cells could induce and/or increase cytolysis of glioma cells that were resistant to NK cells (U251-MG cells) when LAK cells were incubated with BFA prior to exogenous TNF-α treatment. We suggest that intratumoral injection of LAK cells and anti-CD3/antiglioma BFA through an Ommaya reservoir after malignant glioma resection and prior to exogenous TNF-α administration may provide an effective adjuvant therapy against residual malignant glioma.

REFERENCES

1. Grimm EA, Mazumber A, Zhang HZ, et al.: Lymphokine-activated killer cell phenomenon: Lysis of fresh solid tumor cells by interleukin-2-activated autologous human peripheral blood lymphocytes. J Exp Med 155:1823–1841, 1982.
2. Jacobs SK, Wilson DJ, Kornblith PL, et al.: Interleukin-2 and autologous lymphokine-activated killer cells in the treatment of malignant glioma. Preliminary report. J Neurosurg 64:743–749, 1986.
3. Rosenberg SA, Lotze MT, Muul LM, et al.: A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high dose interleukin-2 alone. N Engl J Med 316:889–897, 1987.
4. Barba D, Saris SC, Holder C, et al.: Intratumoral LAK cell and interleukin-2 therapy of human gliomas. J Neurosurg 70:175–182, 1989.
5. Jacobs SK, Wilson DJ, Kornblith PL, et al.: Interleukin-2 or autologous lymphokine-activated killer cell treatment of malignant glioma: Phase I trial. Cancer Res 46:2101–2104, 1986.
6. Bodmer S, Strommer K, Frei K, et al.: Immunosuppression and transforming growth factor-β in glioblastoma. Preferential production of transforming growth factor-β2. J Immunol 143:3222–3229, 1989.
7. Kuppner MC, Hamou MF, Sawamura Y, et al.: Inhibition of lymphocyte function by glioblastoma-derived transforming growth factor β2. J Neurosurg 71:211–217, 1989.
8. Fady C, Gardner AM, Gera JF, et al.: Interferon-induced increase in sensitivity of ovarian cancer targets to lysis by lymphokine-activated killer cells: Selective effects on HER2/*neu*-over expressing cells. Cancer Res 52:764–769, 1992.
9. Naganuma H, Kiessling R, Patarroyo M, et al.: Increased susceptibility of IFN-γ-treated neuroblastoma cells to lysis by lymphokine-activated killer cells: Participation of ICAM-1 induction on target cells. Int J Cancer 47:527–532, 1991.
10. Nitta T, Sato K, Yagita H, et al.: Preliminary trial of specific targeting therapy against malignant glioma. Lancet 335:368–371, 1990.
11. Mizuno M, Yoshida J, Takaoka T, et al.: Reinforced cytotoxicity of lymphokine-activated killer cells toward glioma cells by transfection of the killer cells with the γ-interferon gene. Jpn J Cancer Res 86:95–100, 1995.
12. Takaoka T, Yoshida J, Mizuno M, et al.: Transfection-induced tumor necrosis factor-α increases the susceptibility of human glioma cells to lysis by lymphokine-activated killer cells: Continuous expression of intercellular adhesion molecule-1 on the glioma cells. Jpn J Cancer Res 85:750–755, 1994.
13. Mizuno M, Yoshida J, Takaoka T, et al.: Liposomal transfection of human γ-interferon gene into human glioma cells and adoptive immunotherapy using lymphokine-activated killer cells. J Neurosurg 80:510–514, 1994.
14. Obukhov SK, Gennie MJ, Tutt AL, et al.: The cytotoxic action of lymphokine-activated killer cells upon the human glioma cell line U251 is stimulated by bispecific monoclonal antibody (MoAb) constructs. J Neurooncol 13:203–210, 1992.
15. Yoshida J, Wakabayashi T, Mizuno M, et al.: Clinical effect of intra-arterial tumor necrosis factor-α for malignant glioma. J Neurosurg 77:78–83, 1992.
16. Wakabayashi T, Yoshida J, Seo H, et al.: Characterization of neuroectodermal antigen by a monoclonal antibody and its application in CSF diagnosis of human glioma. J Neurosurg 68:449–455, 1988.
17. Nitta T, Yagita H, Azuma T, et al.: Bispecific $F(ab')_2$ monomer prepared with anti-CD3 and anti-tumor monoclonal antibodies is most potent induction of cytolysis of human T cells. Eur J Immunol 19:1437–1441, 1989.
18. Hook GR, Greenwood MA, Barba D, et al.: Morphology of interleukin-2-stimulated human peripheral blood mononuclear ef-

fector cells killing glioma-derived tumor cells in vitro. J Natl Cancer Inst 80:171–177, 1988.
19. Apasov S, Redegeld F, Sitkosv M: Cell-mediated cytotoxicity: Contact and secreted factors. Curr Opin Immunol 5:404–410, 1993.
20. Ojcius DM, Zheng LM, Sphicas EC, et al.: Subcellular localization of perforin and serine esterase in lymphokine-activated killer cells and cytotoxic T cells by immunogold labeling. J Immunol 146:4427–4432, 1991.
21. Helgason CD, Shi L, Greenberg AH, et al.: DNA fragmentation induced by cytotoxic T lymphocytes can result in target cell death. Exp Cell Res 206:302–310, 1993.
22. Laster SM, Wood JG, Gooding LR: Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. J Immunol 141:2629–2634, 1988.
23. Mizuno M, Yoshida J, Oyama H, et al.: Growth inhibition of glioma cells by liposome-mediated cell transfection with tumor necrosis factor-α gene. Neurol Med Chir (Tokyo) 32:873–876, 1992.
24. Owen Schaub LB, Yonehara S, Crump WL, et al.: DNA fragmentation and cell death is selectively triggered in activated human lymphocytes by Fas antigen engagement. Cell Immunol 140:197–205, 1992.
25. Marlin SD, Springer TA: Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function associated antigen (LFA-1). Cell 51:813–819, 1987.
26. Azuma A, Yagita H, Matsuda H, et al.: Induction of intercellular adhesion molecule 1 on small cell lung carcinoma cell lines by γ-interferon enhances spontaneous and bispecific anti-CD3 × anti-tumor antibody-directed lymphokine-activated killer cell cytotoxicity. Cancer Res 52:4890–4894, 1992.
27. Mahaley MS Jr, Bertsch L, Cush S, et al.: Systemic gamma-interferon therapy for recurrent gliomas. J Neurosurg 69:826–829, 1988.
28. Nitta T, Sato K, Okumura K, et al.: Induction of cytotoxicity in human T cells coated with anti-glioma × anti-CD3 bispecific antibody against human glioma cells. J Neurosurg 72:476–481, 1990.
29. Yoshida J, Wakabayashi T, Mizuno M, et al.: Tumor-specific binding of radiolabelled G-22 monoclonal antibody in glioma patients. Neurol Med Chir (Tokyo) 32:125–129, 1992.
30. Salmeron A, Sanchez-Madrid F, Ursa MA, et al.: A conformational epitope expressed upon association of CD3-ϵ with either CD3-δ or CD3-γ is the main target for recognition by anti-CD3 monoclonal antibodies. J Immunol 147:3047–3052, 1991.
31. Dixon JFP, Law JL, Favero JJ: Activation of human T lymphocyte by cross linking of anti-CD3 monoclonal antibodies. J Leukocyte Biol 46:214–220, 1989.